Advances in Biological Chemistry, 2015, 5, 16-23
Published Online February 2015 in SciRes. http://www.scirp.org/journal/abc
http://dx.doi.org/10.4236/abc.2015.51002

Development of Preparative Chromatography for Proteomic Approach of Mycorrhizal Symbiosis

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Received 23 December 2014; accepted 27 January 2015; published 2 February 2015

Abstract

Although mechanism of symbiosis between arbuscular mycorrhizal fungi (AMF) and host plants has been investigated by genetic analysis, very little knowledge has been obtained because genome analysis of AMF is not perfect yet. Thus, we tried to develop mass purification of proteins using preparative chromatography in order to accelerate roteomic analysis of proteins related to mycorrhizal symbiosis, such as 24 and 53 kDa proteins. In particular, our data showed that 53 kDa proteins would be restrictively expressed when mycorrhizal fungi and host plants were stressed. However, 24 kDa proteins, which appear to be a usable indicator for the existence of various mycorrhizal fungi, were habitually detected in not only AMF but also other mycorrhizal fungi such as ectomycorrhizal fungi (EF). Moreover, we discovered new preparative chromatographical techniques for isolation and mass purification of those proteins. We are convinced that this chromatographical technique will greatly contribute to proteomic approach of mycorrhizal symbiosis.

Keywords

24 kDa Protein, Mycorrhizal Fungi, Preparative Chromatography, Proteomic Analysis, Symbiosis

1. Introduction

Although importance of AMF for sustainable food production is widely known, the mechanism of AMF symbiosis is not so clear. Since AMF have been known to be multinuclear and multiform [1] and to take away cell nuclei from plant hosts [2], it is difficult to approach from genome sequence and often causes confusion in classification of species [3]. Therefore, we focused on proteomic approach for identification of proteins related to

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How to cite this paper: Matsubara, T. and Ishii, T. (2015) Development of Preparative Chromatography for Proteomic Approach of Mycorrhizal Symbiosis. *Advances in Biological Chemistry*, **5**, 16-23. http://dx.doi.org/10.4236/abc.2015.51002

mycorrhizal symbiosis, which would indicate the expression of genes in AMF. Our previous reports show that AMF [4] and orchid mycorrhizal fungi (OMF) [5] have proteins of 24 and 53 kDa in molecular weight that are related to mycorrhizal symbiosis. These facts indicate that various kinds of mycorrhizal fungi will have similar proteins for symbiotic relationship with plants. However, now there are no useful techniques for mass purification of the proteins, which contribute to proteomic approach of mycorrhizal symbiosis.

Therefore, we examined not only separation of proteins related to mycorrhizal symbiosis in AMF and EF using SDS-PAGE techniques, but also development of new preparative chromatography for isolation and mass purification of the proteins on proteomic approach of the symbiosis.

2. Materials and Methods

2.1. SDS-PAGE of Proteins

In this experiment, we used pot-cultured bahiagrass (*Paspalum notatum* Flügge.) roots inoculated with and without *Glomus clarum*, AMF spores, such as *Gigaspora albida*, *Gigaspora margarita* and *G. clarum*, stocked in a refrigerator at 4˚C, and EF hyphae, such as *Tricholoma matsutake* (NBRC 6933) at 4, 25 and 40˚C and *Rhizopogon roseolus* at 25˚C in Ebios media.

Crude proteins were extracted from 5 g of bahiagrass roots inoculated with and without *G. clarum*, approximately 5000 of AMF spores, and approximately 0.1 g of EF hyphae by modified methods of Shannon *et al*. [6]. That is, these samples were homogenized with 0.05 M Tris buffer (pH 7.5) containing 0.05 M NaCl, and then the homogenates were filtered through a cheese cloth. After ammonium sulfate were added into the liquid to 35% saturation, they were stood overnight at 4˚C. They were centrifuged at 8000 g for 30 min and then added ammonium sulfate into the supernatant to 95% saturation. After standing overnight at 4˚C, they were centrifuged at 8000 g for 30 min and then the residues were dissolved in 2 ml of 0.005 M Tris buffer (pH 7.0). Dialyzation was done against 0.005 M Tris buffer (pH 8.0) containing 0.1 M KCl for overnight. After centrifugation at 8000 g for 30 min, we obtained the supernatant with crude proteins.

To detect 24 and 53 kDa proteins in the crude proteins, the SDS-PAGE was carried out by the methods of Laemmli [7]. Each of the crude proteins obtained from bahiagrass roots, *Gi. albida*, *Gi. margarita*, *G. clarum*, *T. matsutake* and *R. roseolus* were used, and the protein bands on the SDS-PAGE gel plates were analyzed by the iMeasure Scan software (iMeasure Inc.).

2.2. Isolation and Mass Purification of Proteins by Chromatography

As shown in **Figure 1**, we developed a new method for isolating and purifying 24 kDa protein related to mycorrhizal symbiosis from crude proteins of soybean (*Glycine max*) roots inoculated with *Glomus intraradices* and sod-cultured peach orchard soil. That is, crude proteins were extracted by modified procedures of Shannon *et al*. [6], and then were separated by medium pressure liquid chromatography (MPLC) with a Diol column to remove ammonium sulfate and to obtain the fractions including 24 and 53 kDa proteins. The solvent was 0.001 M Tris-HCl + 0.001 M NaCl (pH 7.5) and the flow rate was 3 ml/min. A detecter was used at 280 nm. Moreover, we purified these proteins on gradient methods using a preparative high pressure liquid chromatograph (HPLC) with a Diol column.

2.3. Properties of 24 kDa Protein

To analyze the properties of pure 24 kDa protein related to mycorrhizal symbiosis, the protein solution was spotted on thin-layer chromatographic plates, and then various stains, such as ninhydrin, Rhodamine 6G, fluorescein, Dittmer reagent, $FeCl_2$ and hydroxylamine chloride, were sprayed on protein spots. Then, color changes of the spots were observed.

3. Results

Although non-mycorrhizal bahiagrass roots have no 24 and 53 kDa proteins (**Figure 2**), 24 kDa proteins were detected in all of crude proteins extracted from mycorrhizal samples (**Figures 2**, **3** and **4**), but 53 kDa proteins were not detected in crude proteins of *T. matsutake* cultured at 25˚C (**Figure 3**). The 53 kDa proteins were appeared when *T. matsutake* was cultured under low or high temperature stressed conditions such as 4˚C and 40˚C

Roots 40 g

↓

Homogenized with 80 ml of 0.001 M Tris-HCl buffer (pH 7.5) containing 0.001 M NaCl

↓

Soils 100 g

↓

Shook with 200 ml of 0.1 M phosphate buffer (pH 7) containing 0.01 M EDTA and 0.3 M KCl

↓

Extracted crude proteins by modified methods of Shannon *et al.* (1966)
Filtered the homogenates through a cheese cloth.
Added ammonium sulfate into the liquid to 35% saturation.
Stood overnight at 4ºC and then centrifuged at 8000 g for 30 min.
Added ammonium sulfate into the supernatant to 95% saturation.
Stood overnight at 4ºC and then centrifuged at 8000 g for 30 min.
Residues were dissolved in 2 ml of 0.005 M Tris buffer (pH 7.0).

↓

Removed ammonium sulfate and fractionated crude proteins using a MPLC
Column: Diol (75-200 μm) (Fuji Silysia Chemical Ltd.), φ35×400 mm
Solvent: 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)
Flow rate: 3 ml/min, UV: 280 nm

↓

Isolated 24 kDa proteins using a preparative HPLC with a Diol column by gradient methods
Column: Develosil 300 Diol (Nomura Chemical Co., Ltd.), φ10×250 mm
Solvent: 0-10 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)
10-50 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=6.5)
50-60 min 0.001 M Tris-HCl + 0.001 M NaCl (pH=7.5)
Flow rate: 0.5 ml/min, Temperature: 20℃, UV: 280 nm

Figure 1. Procedures for the extraction and purification of proteins in roots and soils.

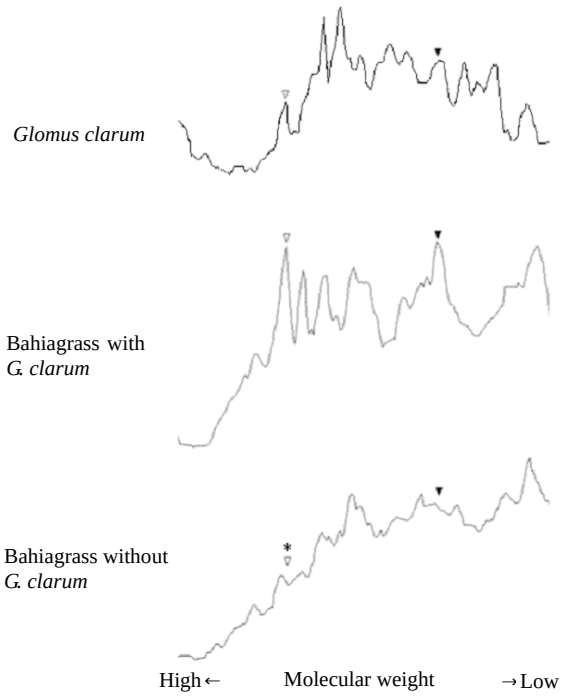

Figure 2. Densitograms of crude proteins extracted from *Glomus clarum* spores and inoculated bahiagrass roots. Bahiagrass roots were harvested on September 6th. White and black arrows show 53 and 24 kDa proteins, respectively. An asterisk shows no significant peak for 53 kDa proteins in bahiagrass without AMF.

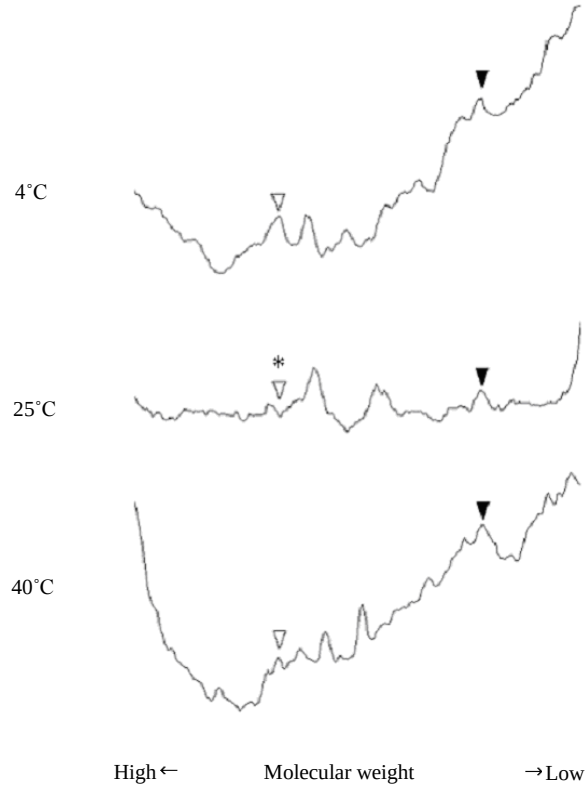

Figure 3. Densitograms of crude proteins *T. matsutake* at 4˚C, 25˚C and 40˚C for 3 days. White and black arrows show 53 and 24 kDa proteins, respectively. An asterisk shows no significant peak for 53 kDa proteins at 25˚C.

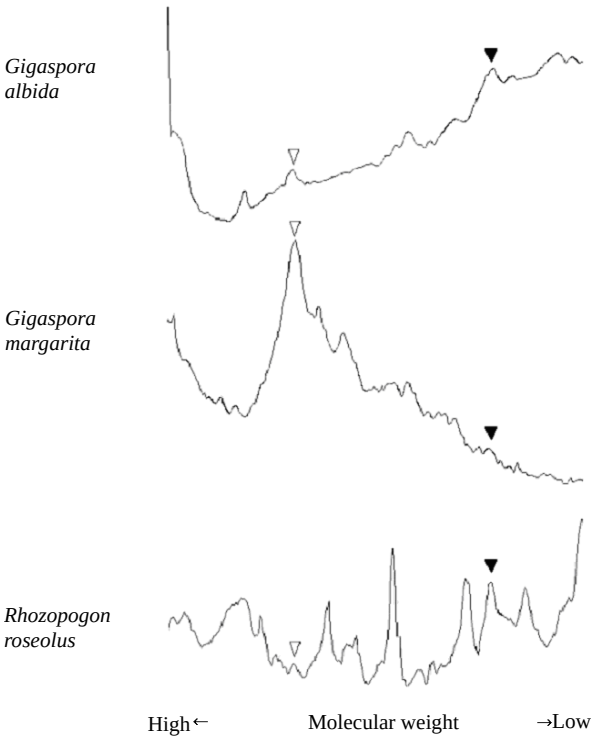

Figure 4. Densitograms of crude proteins extracted from other AMF and ectomycorrhizal fungi. White and black arrows show 53 and 24 kDa proteins, respectively.

(**Figure 3**). The 53 kDa proteins were also detected in pot-cultured bahiagrass roots inoculated with AMF, all AMF spores stored at 4˚C and *R. roseolus* cultured at 25˚C (**Figure 2** and **Figure 4**).

On mass purification of proteins, both of preparative chromatographs, MPLC and HPLC, with a Diol column were very useful for isolating and purifying great amounts of crude proteins extracted by ammonium sulfate precipitation. Moreover, a gradient method on preparative HPLC using a Diol column separated each protein in 24 kDa protein fraction. As shown in **Figure 5(a)**, the chromatogram of soil proteins fractionated by an isochromatic method on a preparative HPLC with a Diol column shows existence of 24 kDa proteins as well as mycorrhizal fungi and plant roots, but it was impossible to isolate pure 24 kDa protein (Peak 5) related to mycorrhizal symbiosis from the 24 kDa protein fraction. The development of a gradient method on preparative HPLC, however, solved the isolation of the same or almost the same as molecular weight of proteins. Thus, it became possible to isolate the pure 24 kDa protein (**Figure 5(b)** and **Figure 6**).

The properties of the 24 kDa protein purified were analyzed using some kinds of stains (**Table 1**). That is, the detection by Rhodamine 6G showed that the 24 kDa protein is bound with lipids. Other stains, such as fluorescein, Dittmer reagent, $FeCl_2$ and hydroxylamine chloride, showed that the lipids were simple ones such as ester lipids, sterol lipids and non-phospholipids. The pink spot made by ninhydrin indicated the existence of peptides. Therefore, the pure 24 kDa protein was lipoproteins with ester and sterol lipids (**Table 1**).

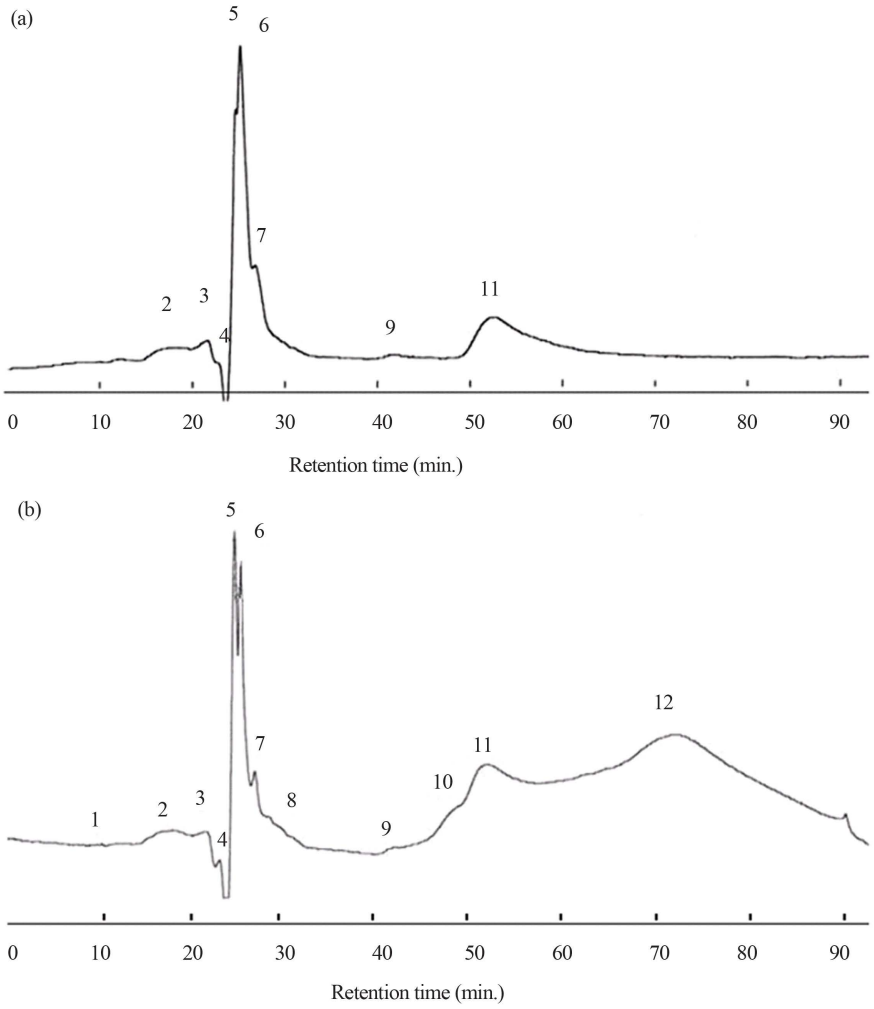

Figure 5. Chromatogram of soil proteins fractionated by HPLC. (a) Isochromatic methods Column: Develosil 300 Diol, φ 10 × 250 mm, Solvent: 0.001 M Tris-HCl + 0.001 M NaCl (pH = 7.5), Flow rate: 0.5 ml/min, Temperature: 20˚C, UV: 280 nm; (b) Gradient methods (see in **Figure 1**).

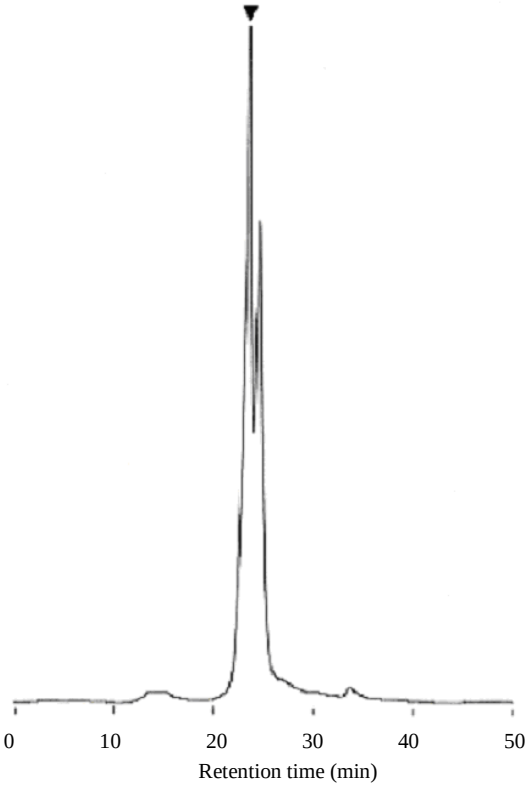

Figure 6. A chromatogram of 24 kDa proteins of *Glomus intraradices*-inoculated soybean roots fractionated by gradient HPLC. Gradient methods: see in **Figure 1**. The black arrow shows 24 kDa proteins in the electrophoresis with SDS plates.

Table 1. The properties of a 24 kDa protein using some kinds of stains.

Stains	Spot color	Decision
Ninhydrin	Slight pink	Amino groups
Rhodamine 6G	Orange	Lipids
Fluorescein	Yellow	Simple lipids
Dittmer reagent	-	Non phospholipids
$FeCl_2$	Purple	Sterol lipids
Hydroxylamine chloride	Slight purple	Ester lipids

4. Discussion

The results of SDS-PAGE indicated that 24 kDa proteins were habitually detected in not only AMF [3], OMF [4] and ericoid mycorrhizal fungi (unpublished), but AMF and EF used. Therefore, the proteins would be related to symbiosis between plants and mycorrhizal fungi. Furthermore, pure 24 kDa protein, which isolated from crude 24 kDa proteins by preparative chromatography, was revealed to be lipoproteins. It suggests that the protein is similar to non-specific lipid transporter proteins with anti-fungal activity reported by Bazghaleh *et al.* [8]. The 24 kDa protein can be related to formation or maintenance of membrane structure of mycorrhizal fungi at inside or around plant roots and protects their hosts from pathogenic fungi.

On the other hand, 53 kDa proteins seem to be expressed only when mycorrhizal fungi and host plants were stressed, because the proteins in *T. matsutake* had appeared at 4˚C and 40˚C. Further, the expression of the pro-

teins in pot-cultured bahiagrass roots may result from water stress in summer. These results suggest that the proteins will be glomalin-like proteins which relieve cells from stresses. Glomalin has been already known as glycoprotein weighs approximately 60 kDa [9], which has similarity to heat shock protein 60 [10].

New creative technique of chromatography developed is very useful for isolation and mass purification of pure 24 kDa protein related to mycorrhizal symbiosis. The protein would contribute to the development of reagents which can detect mycorrhizal fungi by the immunologic analysis. Therefore, sufficient amounts of pure objective protein for building up antibodies are required.

The Diol column has the ability of gel filtration. Thus, our results indicate that MPLC with the Diol column can easily remove ammonium sulfate from large amounts of proteins without conventional dialysis. Moreover, the Diol column in preparative HPLC is effective in isolating single protein from proteins in similar molecular weight under pH gradient conditions. Except for gel filtration of the Diol column, the column would show positive electric charge of silica residue, since this has small amounts of silica without a Diol group. That is, the pH gradient method in range of pH 7.5 - 6.5 causes that silica without the Diol group becomes positively charged, so that retention time of each protein is slightly changed. Although the idea of preparative HPLC in gradient methods for proteins had been already reported by Unger *et al.* [11], the methods have been not utilized. One of the possible reason would be that the pH range used in their experiments was pH 2.0 - 5.0, so that their methods were usable only for proteins stable at low pH. The fact that the pure protein was isolated at pH near 7.0 was an advantage of our methods. Further experiments are needed to develop the utility of the Diol column for protein analysis.

Couto *et al.* [12] have reported that it is difficult to purify proteins from AMF and plants. One of their insistent reasons were difficulty of removing non-protein compounds in plants or fungal tissues. In this investigation, however, these compounds were easily removed from crude proteins by MPLC with the Diol column, and large amounts of the objective proteins were isolated and purified from the crude proteins by gradient preparative HPLC with the Diol column.

AMF are the most important mycorrhizal fungi, because the AMF can colonize almost all of the plants. However, it is very difficult to clarify AMF symbiosis using genome sequence, since it is known that the nuclei of AMF are multiform [1]-[3]. Therefore, proteomic approach of mycorrhizal symbiosis is needed. The new chromatographical techniques developed will greatly contribute to the proteomic approach. In particular, this preparative chromatography would create a new path to analyze proteomically mycorrhizal symbiosis in vitro using the axenic culture of AMF [13] [14].

5. Conclusion

In order to develop preparative chromatography for proteomic approach of mycorrhizal symbiosis, MPLC and gradient preparative HPLC with the Diol column were very useful for mass purification of proteins related to mycorrhizal symbiosis, such as 24 and 53 kDa proteins. The 53 kDa proteins would be restrictively expressed under stress conditions, but the 24 kDa proteins were usually detected in all kinds of mycorrhizal fungi. These chromatographical techniques will serve mycorrhizal studies as well as other proteomic studies.

Acknowledgements

We are grateful to Dr. Chantal Hamel of Saskatchewan University for providing us with soybean seeds and roots.

References

[1] Tisserant, E., *et al*. (2013) Genome of an Arbuscular Mycorrhizal Fungus Provides Insight into the Oldest Plant Symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 20117-20122. http://dx.doi.org/10.1073/pnas.1313452110

[2] Ishii, T., Matsubara, T., Yoneda, M. and Cruz, A.F. (2011) Arbuscular Mycorrhizal Fungi Increase Their Diversities by Taking Away Cell Nuclei from Plant Hosts. *Horticulture Research* (*Japan*), **10**, 59.

[3] Krüger, M., Kruger, C., Walker, C., Stockinger, H. and Schußler, A. (2012) Phylogenetic Reference Data for Systematics and Phytotaxonomy of Arbuscular Mycorrhizal Fungi from Phylum to Species Level. *New Phytologist*, **193**, 970-984. http://dx.doi.org/10.1111/j.1469-8137.2011.03962.x

[4] Ishii, T., Ikeda, T., Rutto, K.L., Cruz, A.F., Matsumoto, I. and Kadoya, K. (1999) Proteins Related to the Mechanism

of Symbiosis between Vesicular-Arbuscular Mycorrhizal Fungi and Plants. *Journal of Japanese Society of Horticultural Science*, **68**, 229.

[5] Matsubara, T., Yoneda, M. and Ishii, T. (2012) Fungal Isolate "KMI" Is a New Type of Orchid Mycorrhizal Fungus. *American Journal of Plant Science*, **3**, 1121-1126. http://dx.doi.org/10.4236/ajps.2012.38135

[6] Shannon, L.M., Kay, E. and Lew, J.Y. (1966) Peroxidase Isozymes from Horseradish Roots. 1. Isolation and Physical Properties. *Journal of Biological Chemistry*, **241**, 166-2172.

[7] Laemmli, U.K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, **227**, 680-685. http://dx.doi.org/10.1038/227680a0

[8] Bazghaleh, N., Hamel, C., Knight, J.D., Gan, Y., Vujanovic, V., Tar'an, B., Cruz, A.F. and Ishii, T. (2012) Phytochemical Regulation of Soil Health Chickpea's Tool Box. *Pulse Cluster Annual Meeting*, Niagara Falls, 5-6 November 2012.

[9] Wright, S.F. and Upadhyaya, A. (1996) Extraction of an Abundant and Unusual Protein from Soil and Comparison with Hyphal Protein of Arbuscular Mycorrhizal Fungi. *Soil Science*, **161**, 575-586. http://dx.doi.org/10.1097/00010694-199609000-00003

[10] Gadkar, V. and Rillig, M.C. (2006) The Arbuscular Mycorrhizal Fungal Protein Glomalin Is a Putative Homolog of Heat Shock Protein 60. *FEMS Microbiology Letters*, **263**, 93-101. http://dx.doi.org/10.1111/j.1574-6968.2006.00412.x

[11] Unger, K.K., Janzen, R. and Jilge, G. (1987) Packings and Stationary Phases for Biopolymer Separations by HPLC. *Chromatographia*, **24**, 144-154. http://dx.doi.org/10.1007/BF02688477

[12] Couto, M.S.R., Paulo, E.L., Wipf, D. and Gaudot, E.D. (2013) Proteomic Studies of Arbuscular Mycorrhizal Associations. *Advances in Biological Chemistry*, **3**, 48-58. http://dx.doi.org/10.4236/abc.2013.31007

[13] Ishii, T. and Horii, S. (2007) Culture of Mycorrhizal Fungi. Patent No. 4979551, Japan Patent Office.

[14] Ishii, T. (2014) The Role and Use of Mycorrhizal Fungi. Rural Culture Association, Tokyo.